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PRINCIPAL INVESTIGATOR: Joshua N. VanHouten

CONTRACTING ORGANIZATION: Yale University
New Haven, CT 06520-8020

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Joshua N. VanHouten

Email: joshua.vanhouten@yale.edu

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Yale University, Internal Medicine, Endocrinology
PO Box 208020
New Haven, CT 06520-8020

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The aims of this research are to determine whether PMCA2 expression correlates with cytosolic calcium levels and sensitivity to apoptosis in human breast cancer cells lines, and whether the absence of PMCA2 alters tumorigenesis or induces drug resistance in a transgenic model of breast cancer. This study found that overexpression of PMCA2 in T47D breast cancer cells reduced the duration of the elevation in intracellular calcium in response to ionomycin treatment. Correlating with the change in intracellular calcium dynamics, T47D cells overexpressing PMCA2 were more resistant than control cells to ionomycin-induced apoptosis. The animal studies are still ongoing. With the caveat that we were not able to show an effect of reducing PMCA2 expression by 80% on apoptosis of MDA-MB-231 breast cancer cells, the data generated thus far support the hypothesis that alterations in intracellular calcium homeostasis may be involved in progression of breast cancer by allowing cells to escape apoptosis. Furthermore, it suggests that targeting molecules involved in this process, such as PMCA2, may be useful in development of new therapeutic compounds.

15. SUBJECT TERMS

intracellular calcium, apoptosis, plasma membrane calcium-ATPase

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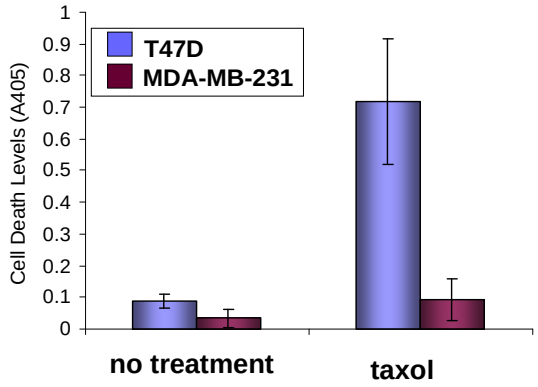
Introduction

The plasma membrane calcium-ATPase isoform 2 (PMCA2) is highly expressed on the apical membrane of mammary epithelial cells during lactation, and is the predominant pump responsible for calcium transport into milk. The lack of PMCA2 causes a low epithelial content in pregnant deafwaddler (dfw-2J) mouse mammary glands due to widespread apoptosis. The apoptosis is likely a result of elevated cytosolic calcium levels due an imbalance of calcium influx and efflux, suggesting that PMCA2 is important not only for calcium transport into milk, but also for maintaining cytosolic calcium levels. In addition, PMCA2 expression correlates with tumor grade, metastases, estrogen-receptor negativity, docetaxol resistance, and poor 5-year survival in human breast cancer. We hypothesize that overexpression of PMCA2 and subsequent enhanced intracellular calcium clearance is a mechanism by which breast cancer cells escape apoptosis. The aims of this research are to determine whether PMCA2 expression correlates with cytosolic calcium levels and sensitivity to apoptosis in human breast cancer cells lines, and whether the absence of PMCA2 alters tumorigenesis or induces drug resistance in a transgenic model of breast cancer.

Body

To determine whether PMCA2 expression protects breast cancer cells from apoptosis, we first analyzed the expression of PMCA2 in MDA-MB-231 and T47D breast cancer cells obtained from ATCC. Expression of PMCA2 is approximately 400-fold higher in MDA-MB-231 cells than in T47D cells. Correlating with the difference in PMCA2



expression, we found that T47D cells were more sensitive to apoptosis induced by taxol (Figure 1).

Figure1. Apoptosis was 2.5-fold higher in T47D cells than MDA-MB-231 cells without treatment, and 8-fold higher with 200 nM taxol. Apoptosis was measured using the Cell Death ELISA-PLUS (Roche, Indianapolis, IN).

To examine the role of PMCA2 in a more rigorous manner, we knocked-down PMCA2 expression in MDA-MB-231 cells and overexpressed PMCA2 in T47D cells. For PMCA2 knockdown studies, the pcDNA™6.2-GW/EmGFP-miR RNAi expression vector kit (Invitrogen, Carlsbad, CA) was used. The sequences encoding the four supplied miRNA's for PMCA2 and the negative control miRNA were cloned using Gateway technology (Invitrogen) into the pT-Rex tet-inducible expression vector, and stably transfected into MDA-MB-231 cells pre-transfected with the pcDNA6-TR vector (encoding the tet transactivator). Although efficient reduction in PMCA2 expression was achieved with the PMCA2-specific miRNA's (Figure 2), reduction of PMCA2 by up to 80% in MDA-MB-231 cells had no effect on apoptosis induced by taxol or ionomycin (Figure 3). This failure could be due to incomplete knockdown of PMCA2. That is, perhaps a 90-

100% reduction of PMCA2 expression is needed to elicit an effect on apoptosis. MDA-MB-231 cells may also express other PMCAs or other molecules involved in intracellular calcium homeostasis that could mask the involvement of PMCA2 in apoptosis. Finally, it is possible that PMCA2 is not involved in apoptosis induced by taxol or ionomycin in MDA-MB-231 cells.

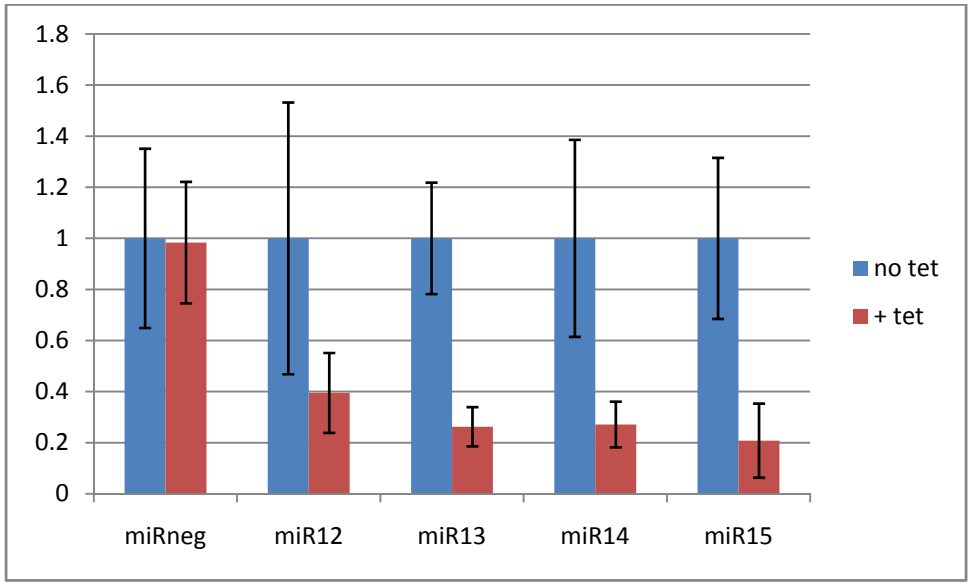


Figure 2. Quantitative RT-PCR confirmed knockdown of PMCA2 expression by 60-80% in MDA-MB-231 cells engineered to express miRNA specific for PMCA2 (miR12-15) in the presence of tetracycline (+tet), compared to negative control miRNA or cells in the absence of tetracycline (no tet).

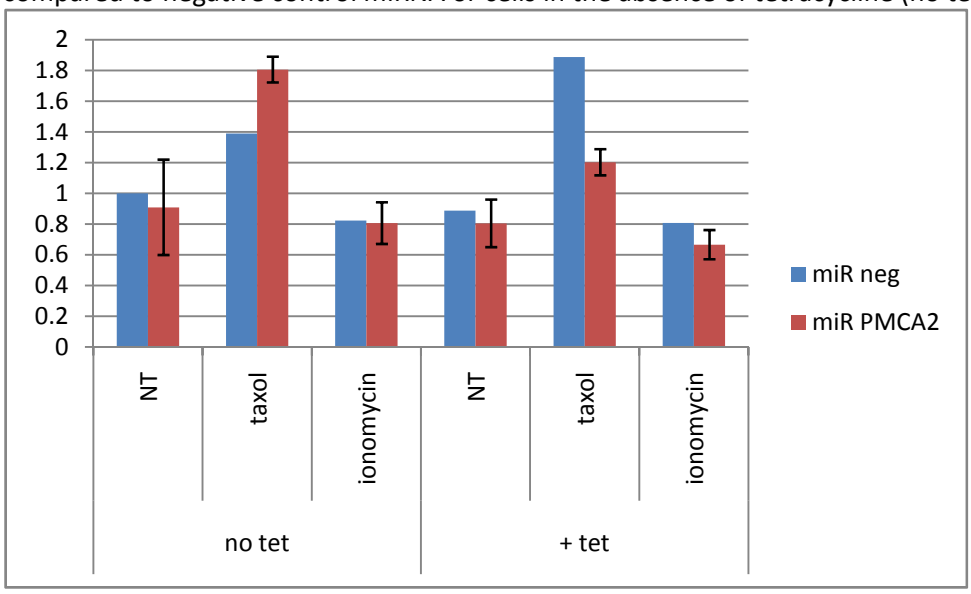


Figure 3. Tetracycline-induced reduction of PMCA2 expression in MDA-MB-231 cells failed to increase apoptosis induced by taxol or ionomycin, as measured by the Cell Death ELISA-PLUS. miR PMCA2 represents the mean value (+/- SEM) for four separate PMCA2-specific miRNAs (miR12-15).

As a complementary approach to knockdown of PMCA2 in MDA-MB-231 cells, we sought to overexpress PMCA2 in T47D cells, which normally have low levels of PMCA2 expression. First PMCA2 was cloned from total RNA isolated from lactating mouse mammary glands using the Phusion High-Fidelity PCR Kit (New England Biolabs, Ipswich, MA). The PCR product was cloned into pENTR/D-TOPO (pENRT/D-TOPO cloning kit, Invitrogen) and transferred to the pT-Rex

vector using Gateway technology (Invitrogen). The entire 3731 bp cDNA clone was sequenced to confirm its identity as authentic mouse PMCA2bw. The sequence of the PMCA2bw cDNA clone is shown in Figure 4.

Mouse PMCA2bw:

ATGGGTGACATGACCAACAGCGACTTTTACTCCAAAAACCAAAGAAATGAGTCGAGCCAT
GGGGGCGAGTTTGGGTGCACCATGGAGGAGCTGCGCTCCCTCATGGAGCTGCGGGGCACC
GAGGCTGTGGTCAAGATCAAGGAGACGTATGGGGACACTGAAGCCATCTGCCGGCGCCTC
AAAACCTCGCCTGTTGAAGGTTTACCAGGCACTGCTCCAGACTTGGAAAAGAGGAAACAG
ATTTTTGGGCAAAACTTCATACCTCCAAAGAAACCAAAAACCTTCCTGCAGCTGGTGTGG
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CTGTCCTTCTACCACCCACCGGGAGAGAGCAATGAAGGATGTGCCACGGCCCAGGGTGGG
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GTCATCTGTGTGGTCCTGGTCACAGCCTTCAATGACTGGAGTAAGGAGAAACAGTTCCGG
GGCCTGCAGAGCCGAATTGAGCGGGAGCAGAAGTTCACTGTGGTCCGGGCCGGCCAGGTG
GTTCAGATCCCTGTGGCCGAGATCGTGGTCGGGGACATTGCCCAGATCAAATATGGTGAC
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CCTCTGAAGAGTGCAGAGGGCGGCGATGCAGATGACAAGAAGAAAGCCAACATGCACAAG
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GTCAAGTTCTTCATCATTGGTGTGACGGTGCTGGTGGTCGCTGTGCCCGAGGGCCTCCCT
CTGGCTGTCACCATCTCACTGGCCTATTCTGTGAAGAAAATGATGAAGGACAACAACCTG
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TACAAGGAGATCCCCGATCCCAGCTCCATCAATGCCAAGACGCTGGAGCTGCTGGTCAAC
GCCATTGCCATCAACAGCGCCTACACCACCAAGATCCTTCCCCCAGAAAAAGAGGGAGCC
CTGCCCCGGCAGGTGGGCAACAAGACAGAGTGCGGCCTGCTGGGCTTTGTGCTGGACTTG
AGGCAGGACTACGAGCCGGTGCGCAGCCAGATGCCAGAGGAGAAGCTGTATAAGGTGTAC
ACCTTCAACTCCGTGCGCAAGTCCATGAGCACCGTCATCAAGATGCCCGACGAGAGCTTC
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GGGGCAGGGGAAGCCCGTGTCTTCCGGCCCCGAGACAGGGATGAGATGGTTAAGAAGGTG
ATCGAGCCCATGGCCTGTGACGGGCTCCGTACCATCTGCGTGGCCTATCGTGACTTCCCC
AGCAGCCCTGAGCCTGACTGGGACAATGAGAATGACATTCTCAATGAACTCACGTGCATC
TGCGTGGTGGGCATCGAAGACCCAGTACGACCTGAGGTCCCAGAAGCCATCCGCAAGTGC
CAGCGGGCAGGTATCACAGTCCGCATGGTCACCGGTGACAATATCAACACAGCCCGGGCC
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GAATTCAATCGGAGGATTCGCAACGAGAAGGGGGAGATTGAGCAGGATCGGATTGACAAG
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GGCACAGATGTGGCCAAGGAGGCCTCAGACATCATCCTGACAGATGACAACTTCAGCAGC
ATCGTCAAGGCAGTGATGTGGGGCCGTAACGTCTATGACAGCATATCCAAATTCCTGCAG
TTCCAGCTGACTGTCAACGTGGTGGCGGTGATCGTGGCCTTCACGGGCGCCTGCATTACA
CAGGACTCCCCTCTCAAGGCTGTGCAGATGCTCTGGGTGAACCTCATCATGGACACGTTT
GCCTCCCTGGCCCTGGCCACAGAGCCACCTACGGAGACTCTGCTTCTGAGGAAACCGTAC
GGTCGCAACAAGCCGCTCATCTCGAGGACCATGATGAAGAACATCCTGGGCCACGCCGTC
TACCAGCTCACCCTCATCTTCACCCTGCTCTTCGTGGGTGAGAAGATGTTCCAGATCGAC
AGCGGAAGGAACGCCCCGCTGCACTCACCACCCTCAGAGCACTACACCATCATCTTCAAC
ACCTTCGTCATGATGCAGCTTTTCAACGAGATCAACGCCCGCAAGATCCACGGCGAGCGC
AACGTCTTTGACGGGATCTTCCGGAACCCCATCTTCTGCACCATCGTTCTTGGCACTTTC
GCCATCCAGATAGTGATCGTGCAGTTTGGCGGGAAGCCCTTCAGCTGCTCCCCACTCCAG

CTGGACCAGTGGATGTGGTGCATCTTCATAGGCCTGGGAGAGCTCGTCTGGGGCCAGGTC
ATCGCCACCATCCCTACCAGCAGGCTCAAGTTCCTGAAAGAGGCAGGGCGTCTAACACAG
AAGGAGGAGATCCCCGAGGAGGAGTTAAATGAGGATGTGGAAGAGATAGACCACGCAGAG
CGGGAGCTTCGCCGTGGCCAGATCCTGTGGTTCCGGGGCCTGAATCGGATCCAGACACAG
ATCCGCGTCGTGAAGGCGTTCCGTAGCTCTCTCTATGAAGGGTTAGAAAAACCCGAGTCT
CGAACCTCCATCCATAACTTCATGGCTCATCCTGAATTCCGGATCGAAGATTCCCAGCCC
CACATCCCCCTCATCGATGACACCGACCTGGAAGAAGATGCCGCGCTCAAGCAGAACTCG
AGCCCGCCGTCCTCGCTCAACAAGAACAATAGCGCCATCGACAGCGGGATCAACCTGACG
ACCGACACGAGCAAATCAGCTACCTCTTCAAGTCCAGGGAGCCCCATCCACAGCCTGGAG
ACGTCGCTTTAG

Figure 4. Sequence of PMCA2bw cloned from the lactating mouse mammary gland.

Mouse PMCA2bw, in the pT-Rex vector, or pT-Rex empty control vector, were stably transfected into T47D cells and the cells were treated with ionomycin or taxol overnight. After treatment, apoptosis was analyzed using the Cell Death ELISA-PLUS. No difference was seen between T47D/control and T47D/PMCA2 cells treated with taxol (Figure 5). However, PMCA2-overexpressing T47D cells were more resistant to ionomycin-induced apoptosis than the control cells (Figure 6). High extracellular calcium increased the rate of apoptosis induced by ionomycin, while EGTA reduced apotosis, except at very high extracellular calcium levels (Figure 7). Furthermore, PMCA2 overexpression reduced apoptosis at 2 and 10 mM extracellular calcium, but had little effect when no extracellular cacium was present (Figure 7).

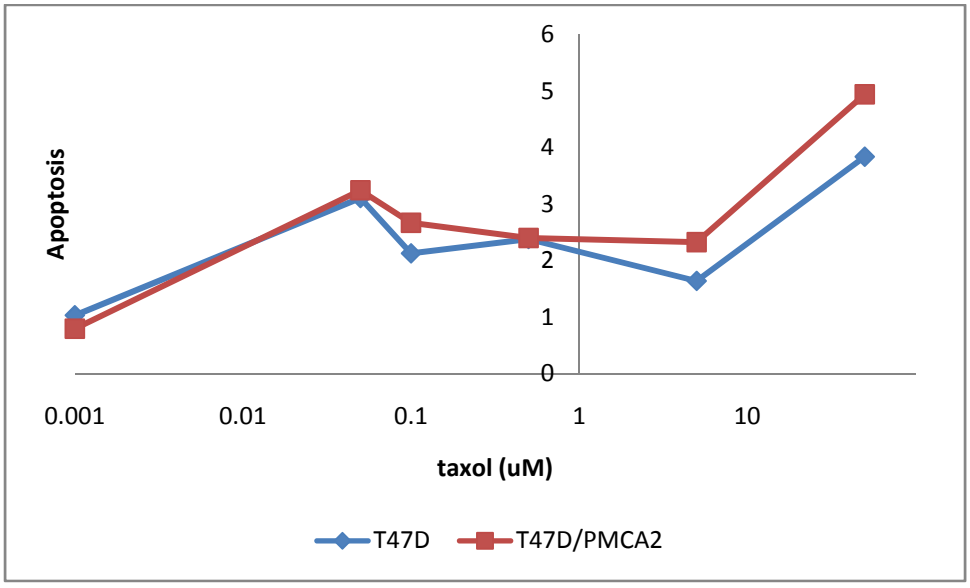

Figure 5. Apoptosis was similar in T47D (control) cells and T47D/PMCA2 (PMCA2 overexpressing) cells when treated with varying concentrations of taxol overnight.

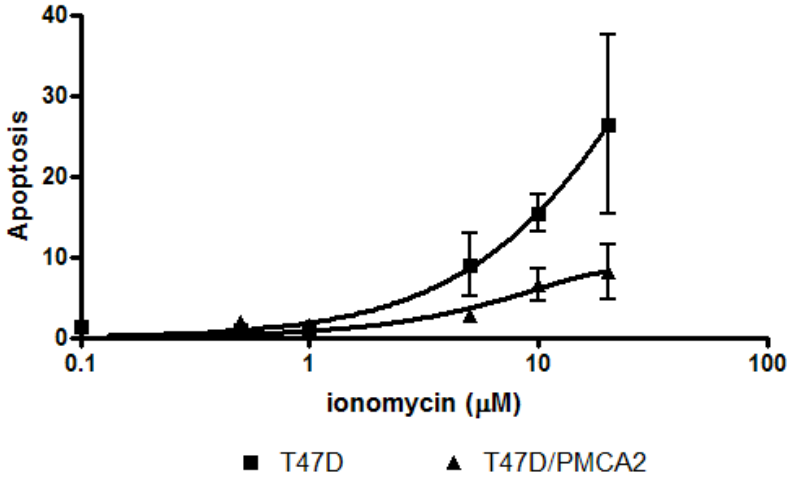

Figure 6. PMCA2 overexpression reduced apoptosis induced by ionomycin in T47D cells. For each concentration of ionomycin, the mean +/- SEM is shown. The difference in ionomycin-induced apoptosis between control cells and T47D/PMCA2 cells was significant by the comparison of fits test (F-test) The F-test showed a 0.92% chance that both data sets fall on the same curve, but a 99.08% chance that the data sets fall on different curves.

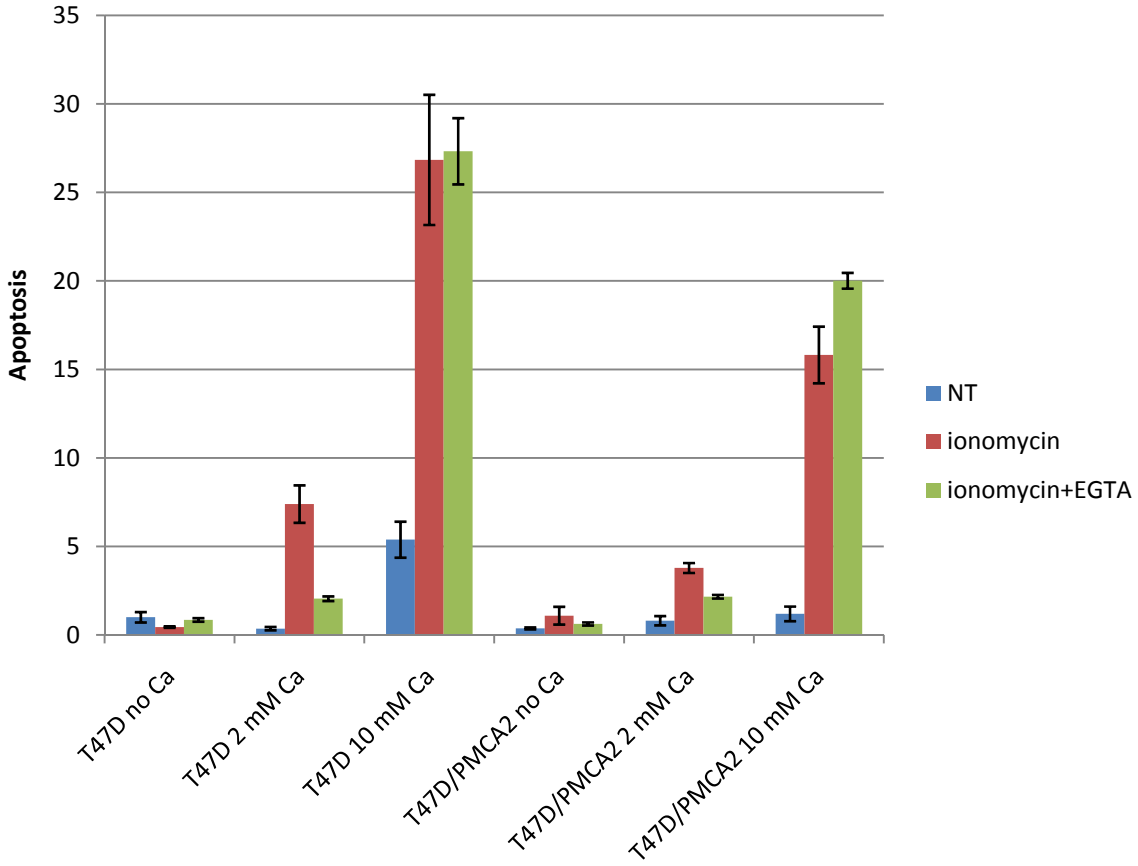

Figure 7. Treatment with 10μM ionomycin induced apoptosis of T47D cells only in the presence of extracellular calcium, and the magnitude of the apoptotic response depended on the concentration of extracellular calcium. 5mM EGTA inhibited apoptosis at 2mM extracellular calcium, but not at 10mM extracellular calcium. In the presence of extracellular calcium, PMCA2 reduced apoptosis induced by

ionomycin ($p<0.005$ at 2mM and $p<0.05$ at 10mM calcium by Student's t-test). Bars represent the mean +/- SEM of three experiments. Samples were run in quadruplicate in each experiment.

The results above demonstrate that PMCA2 overexpression protects T47D cells from apoptosis induced by ionomycin. The action of ionomycin is to allow extracellular calcium to enter the cell. Our hypothesis is that higher levels of PMCA2 allow the cells to remove more of the entering calcium, and therefore protect the cells from toxicity associated with sustained elevations of intracellular calcium. This hypothesis predicts that intracellular calcium would be lower in T47D cells overexpressing PMCA2 than in control cells. To test this prediction, we loaded T47D control cells and T47D/PMCA2 cells with 2μM fluo-4 (Invitrogen) at room temperature for 1 hour. After washing the cells twice and incubating at room temperature for another 30 minutes, the cells were perfused with media containing 10mM calcium for 2 minutes to obtain a baseline reading, then with 10mM calcium plus 5μM ionomycin for about 10 minutes, followed by 10mM calcium without ionomycin for about 10 minutes, and finally calcium-free media. Cells were imaged by confocal microscopy using a Zeiss LSM510 confocal microscope once every 1-2 seconds. In control T47D cells, ionomycin induced a rapid increase in intracellular calcium that persisted even on removal of ionomycin from the media (Figure 8). In contrast T47D/PMCA2 cells displayed a transient increase in intracellular calcium upon addition of ionomycin, and intracellular calcium fell to below baseline levels when the calcium-free media was perfused. In calcium-free media, ionomycin induced a rapid, transient increase in intracellular calcium in T47D control cells and PMCA2-overexpressing cells, but the magnitude of the increase was smaller in T47D/PMCA2 cells. The transient rise results from release of calcium from intracellular stores (endoplasmic reticulum and mitochondria). The affect of PMCA2 overexpression on intracellular calcium dynamics supports the idea that PMCA2 may protect T47D cells from ionomycin-induced apoptosis by preventing intracellular calcium overload. Further support for this idea comes from the observation of plasma membrane blebbing in the very same T47D control cells with the highest sustained levels of intracellular calcium in the live cell imaging experiments (Figure 9). Membrane blebbing is a classic hallmark, and early indicator of apoptosis. No membrane blebbing was observed in T47D/PMCA2 cells during the live cell imaging studies.

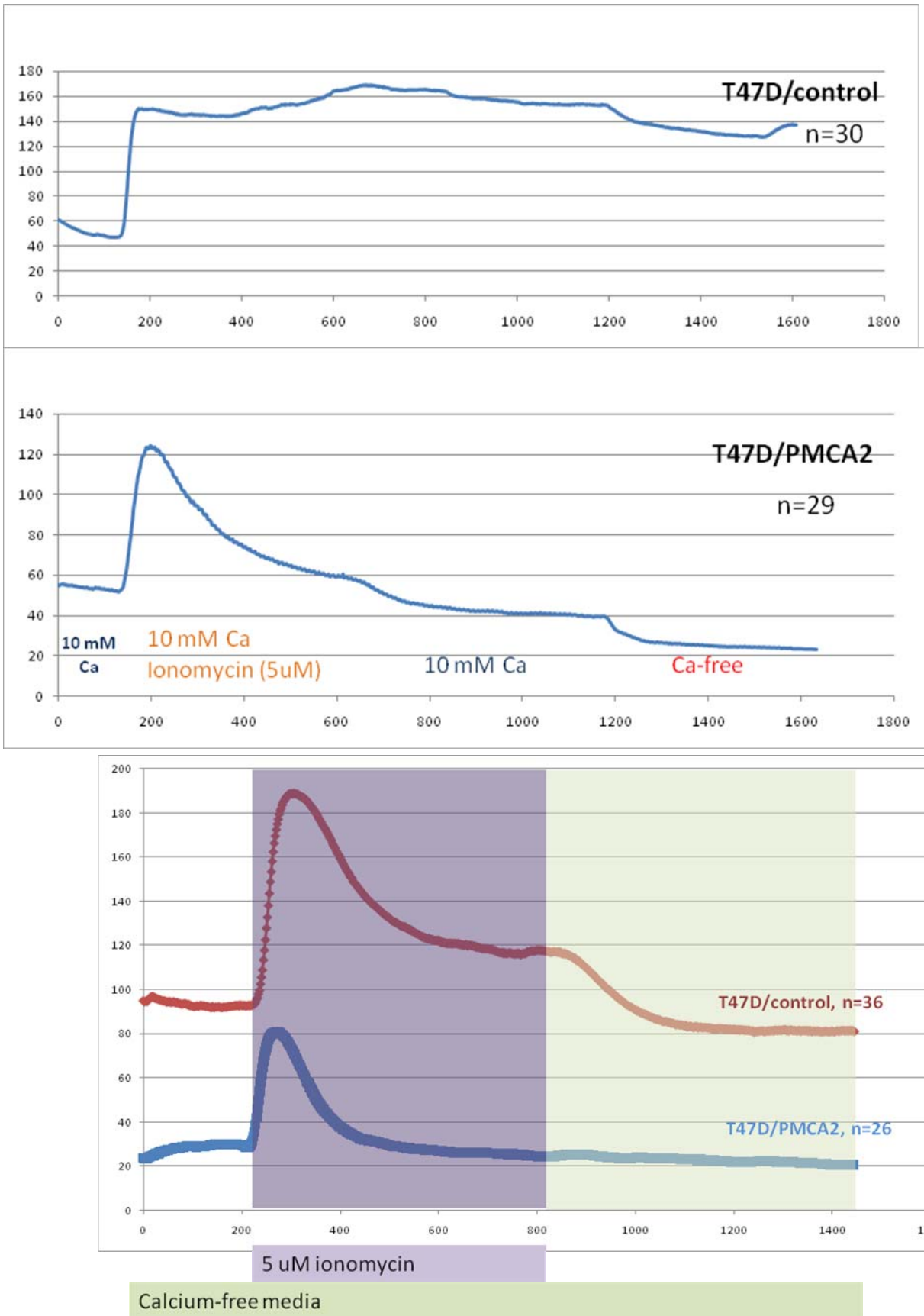

Figure 8. Top: Fluo-4 was used to image intracellular calcium in T47D/control cells and T47D/PMCA2 cells. Fluorescence intensity is on the y-axis, while time (in seconds) is shown on the x-axis. The experiment shown on the top is a representative result showing the sustained increase in intracellular

calcium in T47D/control cells after addition of ionomycin, but transient increase in intracellular calcium in T47D/PMCA2 cells. Similar results were obtained in three separate experiments. The bottom panel shows that extracellular calcium influx is necessary for the sustained increase in intracellular calcium in T47D/control cells. The transient increase upon addition of ionomycin in calcium-free media is due to release of intracellular calcium stores followed by equilibration of intracellular and extracellular calcium.

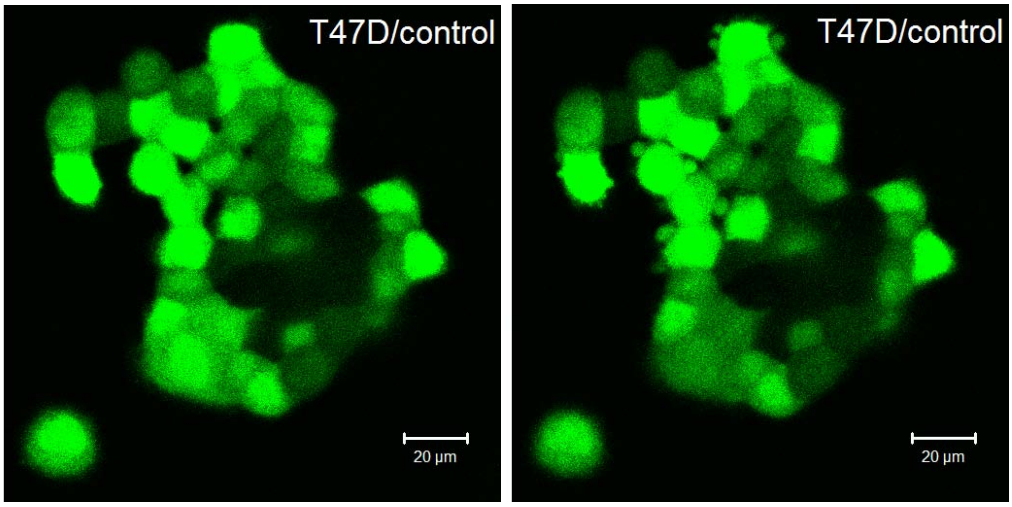

Figure 9. Membrane blebbing was observed in cells with high intracellular calcium (bright green, fluo-4 indicator) after about 7 minutes of ionomycin treatment at 10 mM extracellular calcium. A representative field of cells is shown before (left) and just after (right) blebbing was observed.

Another goal of this research is to determine whether the absence of PMCA2 affects the development, growth, or resistance to treatment of mammary tumors in a mouse model of breast cancer. We obtained dfw-2J mice from The Jackson Laboratory (these mice lack PMCA2) and crossed these mice with MMTV-neu mice (also from The Jackson Laboratory, Bar Harbor, ME). We are currently generating a cohort of dfw-2J homozygous/MMTV-neu mice for these studies.

Key Research Accomplishments

-cloned mouse PMCA2bw from mouse mammary gland

-generated T47D cells that overexpress PMCA2

-PMCA2 overexpression protects T47D cells from apoptosis induced by ionomycin (but not taxol)

-PMCA2 overexpression alters intracellular calcium response to ionomycin, correlated with membrane blebbing (an indicator of apoptosis)

Reportable Outcomes

-mouse PMCA2bw cDNA clone

-MDA-MB-231 cells with a) pcDNA6/TR and /or pT-Rex/miRNA's (as described in text)

-T47D/PMCA2 cells (stably overexpress PMCA2)

Conclusion

PMCA2 overexpression altered intracellular calcium dynamics and protected T47D cells from apoptosis in response to ionomycin. These results support the idea that PMCA2 or other molecules involved in intracellular calcium homeostasis could impact apoptosis in breast cancer cells. Because escape from apoptosis is an important step in progression of cancer cells, compounds or drugs that inhibit PMCA2 or similar molecules in the calcium

removal/sequestration pathway represent potential new therapies. The cell line developed in this study could be used as a screening tool to identify compounds that inhibit PMCA2, using intracellular calcium as a readout.

References:

None

Appendices:

None

Supporting Data

None